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13. ABSTRACT (Maximum 200 Words) The object of this project is to develop a high-throughput method for screening potential inhibitors of breast cancer cell haptotaxis and chemotaxis, and to apply this method to identify signaling events mediating constitutive migration of malignant breast cells. The pathways that control these signaling events may be targets for development of new classes of anti-tumor drugs. The significant advances made during the third year of the project include generation of laminin-1 fragments for examining-integrin-specific adhesion and signaling, and identification of rho-A as a target for the migration inhibitory compound perillyl alcohol. The significance of this work is demonstration of the utility of the novel migration inhibitor drug screen we have developed, plus development of reagents that will enable us to examine the signaling associated with specific integrin complexes in breast cells.				
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Table of Contents

Cover Page	1
Form 298	2
Table of Contents	3
Introduction	4
Body	4
Key Research Accomplishments	8
Reportable Outcomes	8
Conclusions	9
References	10
Appendices	10

Introduction

The purpose of this project is to identify the mechanisms governing the constitutive migration of breast cancer cells, with an eye towards reducing the severity of malignant breast cancer by inhibiting the signaling pathways responsible for maintaining this migration. The approaches proposed in this project focus on developing an in vitro migration assay suitable for identifying new biochemical inhibitors of tumor cell migration, and to characterize the mechanism of action of cell migration inhibitors. As suggested in the Review of the last Annual Review, we have revised the Statement of Work to more accurately reflect the activities undertaken in the past year and our plans for the fourth and final year of the project.

Body

The goals of this project have been defined in two specific aims. The progress in each Aim is described below.

Specific Aim 1: Define the components of the pertussis toxin-sensitive G protein signalling pathway that mediates migration of normal and malignant breast cells on laminin-5.

Specific elements of this aim that were to be addressed in the first 12 months of this project are:

Task 1. Optimize assays for identifying signalling proteins in migrating cells, Months 1-6.

In the course of developing a migration assay, we discovered that intracellular Ca^{+2} levels are directly proportional to migration rate in our cells, and that both of these factors vary considerably on different extracellular matrix proteins. We have focused our attention on following up on these findings.

- a. Develop protein kinase and lipid kinase assays using whole cell lysates as starting material

RACK1: As previously reported in the 2000 annual report, we submitted a manuscript characterizing RACK1 inhibition of migration in CHO cells. This manuscript has since been published in Journal of Cell Science.

FAK/CAS: We have completed construction of the recombinant, his-tagged laminin-5 $\gamma 2$ chain that represents the truncated form after collagenase cleavage. Our plan is to transfect this into laminin-5 expressing cells (e.g., MCF-10A, 804G) and to isolate both full length and recombinant/truncated forms by affinity chromatography. We have not yet been able to successfully express the $\gamma 2$ construct in cells.

HETEROTRIMERIC G PROTEINS:

This work was published in the past year. No further work on G proteins is planned.

- b. Optimize methods for isolating integrin complexes

Progress: This project was abandoned after the first year.

- c. Optimize sensitivity and throughput of non-fluorescence, dye-based cell migration assay.

Progress: This project is complete.

Task 2. Identify integrin-associated signalling proteins in breast cells migrating on laminin-5, Months 6-30.

Progress: Much of the emphasis of this project has turned to examining the roles of specific signaling proteins (FAK, CAS, G proteins) and the work in progress is discussed above.

In addition, we have turned our attention to capitalizing on our finding that breast cells require Ca^{+2} to migrate, and this has spawned the development of a new project, described below:

Model for investigating integrin communication on laminin-1.

To determine the binding properties of MCF-10A cells on laminin-1, cells were pre-incubated with function blocking antibodies directed against integrins expressed by MCF-10A cells (data not shown), a chelator of cytosolic calcium (BAPTA-AM) and the PI-3 kinase inhibitor wortmannin. Cells were also incubated in heparin sulfate to demonstrate that adhesion to laminin-1 is not dependent upon non-specific heparan charged interactions. Blocking integrins $\alpha 1$, $\alpha 6$, and $\beta 1$ inhibit adhesion of MCF-10A cells to laminin-1 indicating that these are the primary integrins that determine the strength of adhesion to laminin-1 (Fig. 1). $\alpha 6$, which can form a heterodimer with $\beta 4$, are binding laminin-1 in the $\alpha 6\beta 1$ configuration as blocking $\beta 4$ has no effect on adhesion (Fig 1. Lane 14). Although not statistically significant, blocking $\alpha 3$ appears to increase the strength of MCF-10A cell adhesion to laminin-1. Integrin $\alpha 3$ is included in these studies as a potential laminin-1 binding molecule due to reports that $\alpha 3$ acts as a negative regulator of $\alpha 6$ activity in other cell lines. MCF-10A engagement to laminin-1 is dependent upon cytosolic calcium as pre-incubation with BAPTA-AM abolishes adhesion (Fig. 1, lane 3). Integrin mediated release of cytosolic calcium is partially dependent upon the activity of PI-3 Kinase as wortmannin, a specific inhibitor of PI-3 Kinase at the concentrations used, blocks adhesion in a concentration dependent manner (Fig. 1, lanes 4,5). Heparin sulfate did not block adhesion, demonstrating that MCF-10A adhesion to laminin-1 is not dependent upon proteoglycan-heparan interactions.

We have selected six domains of laminin-1 that have a high probability of being a substrate for integrins $\alpha 1$, $\alpha 3$, and $\alpha 6$. Because glycosylation and conformation are important for regulating integrin affinity, these fragments of laminin-1 are being produced in an insect cell expression system. The late viral promoter gp64 was used, and each domain was appended with a secretion signal peptide and a 6X polyhistidine tag. Purification was accomplished with metal-chelate anion chromatography. The major problem we have encountered in achieving this task is the low yield of protein in our expression system. While the baculovirus system generally achieves a yield of 1-2 mg/L of protein, the yield of our proteins ranges from 30-300 $\mu\text{g/L}$. This result is a combination of the following factors: secreted proteins generally provide lower yield, late viral promoters provide lower yield than very late viral promoters, and small protein size.

We have successfully expressed each domain and have tested them for their ability to support adhesion of MCF-10A cells. In order to screen each recombinant protein as an adhesive substrate for epithelial cells, MCF-10A cells were plated in wells coated with purified proteins and intact laminin-1. Due to low concentration of recombinant proteins, wells were coated with equimolar amounts of protein (21.1nM), and not identical masses. Statistically reliable adhesion to

recombinant proteins by MCF-10A cells was not demonstrated (Fig. 2). Manganese, which acts to strengthen integrin engagement, did not influence MCF-10A adhesion to the recombinant proteins, and no recombinant protein, when pre-incubated with MCF-10A cells, influenced adhesion to laminin-1 (data not shown). In order to determine if the failure to bind the recombinant domains was cell line-specific, MDA-MB-231 cells, which bind to laminin-1 more strongly than MCF-10A cells were also tested for adhesion. As indicated by pre-incubation with function blocking anti-integrin antibodies, MDA-MB-231 cells engaged laminin-1 with the $\alpha 6 \beta 1$ integrin (Fig. 2, lanes 2-8). These cells did not, however, bind to proteins A6-G4 any greater than negative control (Fig. 2 lanes 9-13). These cells did show significant adhesion to protein G5, although in an integrin-independent manner (Fig 2, Lanes 14-19). Curiously, MDA-MB-231 adhesion to laminin-1 was also independent of proteoglycan-heparan charged interactions (Fig 2, lane 20). This indicates that these cells engage protein G5 via a member of another family of adhesion receptors, such as E-cadherin, a-dystroglycan, or CD44.

Because epithelial cell adhesion to substrate normally results in formation of focal complexes and spreading, MDA-MB-231 cells were incubated on laminin-1 and protein G5 for 90 minutes, and visualized by fluorescence microscopy. Focal complex formation was visualized indirectly with anti-vinculin antibody, and cell structure was visualized by staining the actin cytoskeleton with FITC-conjugated phalloidin (Fig. 3). MDA-MB-231 cells spread and formed distinct focal complexes on laminin-1 (Fig. 3, panel A,B). On protein G5, adherent cells remained rounded and did not form any distinct cytoskeletal structures or focal complexes (Fig. 3, panels C,D).

From these data we conclude that these fragments do not support strong adhesion to the two breast cell lines tested, though they are glycosylated and likely are presented in a native conformation. Further work will be required to resolve the poor adhesion to these fragments.

Task 3. Identify inhibitory compounds that block migration of malignant breast cells on laminin-5, Months 12-30.

- a. Optimize assay. This is completed.
- b. Screen inhibitors of biochemical signalling pathways in migration assays.

We have been unable to overcome our legal troubles with Polyfiltronics, and the 96 well plate we developed has not been produced, and likely will not be pursued further. We have instead turned our attention to characterizing the migration inhibiting properties of perillyl alcohol, which we identified as a non-lethal inhibitor of MCF-10A cell migration during the development of our migration assay (see below).

Specific Aim 2: Identify the specific protein targets of compounds that inhibit migration of normal and malignant breast cells on laminin-5.

We have focused our attention on characterizing the mechanism of action of perillyl alcohol. This project will be submitted for publication in the next month. A summary of the submitted work is as follows:

Perillyl alcohol (POH) is currently in clinical trials for prevention and treatment of various cancers. One of the identified cellular activities of perillyl alcohol is the inhibition of the enzyme type 1 geranylgeranyl transferase. RhoA is a protein that requires prenylation to be active and to initiate a pathway involved in cell migration. We hypothesized that the decreased migration

observed in POH-treated MCF-10A and MDA-MB 435 cells would be the result of the translocation of RhoA to the cytosol, thus impairing its migration signaling pathway. In this study, we first measured by western blot whether exposure to perillyl alcohol down-regulated the production of RhoA protein in human breast non-tumorigenic MCF-10A and MDA-MB 435 breast cancer cells and found that it did not. We also found that RhoA protein expression was greater in the non-cancerous MCF-10A cells than the MDA-MB 435 cells. Next the distribution of RhoA protein between the aqueous and detergent-enriched phases was analyzed by western blots from cell lysates of MCF-10A cells and MDA-MB 435 human breast cancer cells. At 0 hours of exposure, all RhoA protein was located in the detergent-enriched phase following treatment with 0.5 mM perillyl alcohol in MCF-10A cells. However, after 18 hours of exposure RhoA protein accumulated in the aqueous phase in MCF-10A cells. For MDA-MB 435 cells even at 0 hours of treatment with 0.3 mM perillyl alcohol RhoA accumulated in the aqueous phase, which was again detected after 18 hours of exposure. The morphological effects of perillyl alcohol were investigated in MCF-10A cells and MDA-MB 435 cells following an 18-hour exposure to 0.5 mM or 0.3 mM, respectively. Perillyl alcohol treated MCF-10A and MDA-MB 435 cells showed a complete disassembly of stress fiber and focal adhesion complex formation, consistent with a loss of RhoA protein function.

Revised Statement of Work:

Task 1. Optimize assays for identifying signaling proteins in migrating cells, Months 1-12.

- a. Select materials and format for automated migration assay.
- b. Develop and optimize sensitivity and throughput of non-fluorescence, dye-based assay.
- c. Apply assay to characterize known anti-tumor drugs.

Task 2. Identify integrin-associated signaling events controlling breast cell migration on laminins, Months 13-36.

- a. Apply Ca^{+2} signaling assays to normal and malignant breast cells on laminin-1 and -5.
- b. Apply cAMP signaling assays to normal cells plated on laminin-5 in the presence or absence of integrin-stimulating antibody.
- c. Identify pertussis toxin-sensitive proteins in migrating normal breast cells plated on laminin-5.

Task 3. Identify inhibitory compounds that block migration of malignant breast cells on fibronectin, Months 12-36.

- a. Optimize sensitivity and throughput of fluorescence-based cell migration assay.
- b. Screen inhibitors of biochemical signalling pathways in migration assays.
- c. Determine mechanism of action of inhibitors at protein/second messenger level.

Task 4. Generate recombinant fragments of laminin-1, Months 24-40.

- a. Construct expression plasmids for use in baculovirus expression system.
- b. Infect insect cells, isolate protein fragments.
- c. Characterize purified fragments: glycosylation, adhesion promoting activity, integrin binding, etc.
- d. Determine migration activity of normal and malignant cells on fragments.

Task 5. Examine integrin-specific signaling in isolated laminin-1 fragments, Months 36-48.

- a. Microscopy: Effects on cell shape, focal adhesion formation, cytoskeletal rearrangements.
- b. Calcium signaling: Examine calcium flux on fragments.
- c. Other signaling: FAK Phosphorylation, phosphatidyl inositol-3 kinase activity, rho/rac GTP binding activity.

Appendices

key research accomplishments

- Completion of the design and small scale manufacture of 96-well fluorescence-based cell migration plates
- Identification of RACK1 as an inhibitor of CHO cell migration.
- Identification of FAK as a laminin-5 signaling protein in human breast cells
- Identification of Ca²⁺ as an essential component of cell migration signaling in breast cells
- Identification of perillyl alcohol as a non-cytotoxic inhibitor of breast cell migration
- Determination that perillyl alcohol inhibits RhoA membrane association in breast cells

Reportable Outcomes:

manuscripts

Plopper, G.E., J.L. Huff, W.L. Rust, M.A. Schwartz, and V. Quaranta. Antibody induced activation of $\beta 1$ integrin receptors stimulates cAMP-dependent migration of breast cells on laminin-5. Molecular Cell Biology Research Communications, 4:129-135, 2000.

Buensuceso, C.S., D. Woodside, J.L.Huff, G.E. Plopper, and T.E. O'Toole. The WD protein Rack1 mediates PKC and integrin dependent cell migration. Journal of Cell Science, 114:1691-1698, 2001.

abstracts

None

presentations

None.

patents and licenses applied for and/or issued

none

degrees obtained that are supported by this award

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development of cell lines, tissue or serum repositories

N/A

informatics such as databases and animal models, etc.

N/A

funding applied for based on work supported by this award

none

employment or research opportunities applied for and/or received based on experiences/training supported by this award

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Conclusions.

In this report we demonstrate the successful application of a novel anti-migration assay we have developed to establish that perillyl alcohol inhibits the migration of normal and malignant cells at concentrations that are non-toxic to normal cells. We also have generated important research tools (insect plasmids encoding secreted forms of laminin-1 fragments) that will enable us to carefully examine the migratory signaling that arises from specific integrin receptors bound to a native substrate. Our major limitations at this point are finding a source of funding to conduct a large-scale screen of other potential migration inhibiting compounds, and the relatively poor yield of the laminin fragments

that slows our progress. The knowledge generated in this project is useful to the pursuit of anticancer treatments because it demonstrates an effective method for rapidly identifying anti-migratory drugs that are non-toxic. This project has also allowed us to identify key molecular players that contribute to the migration of cells and thus may serve as molecular targets of new therapies.

References.

None cited.

Appendices.

See attached.

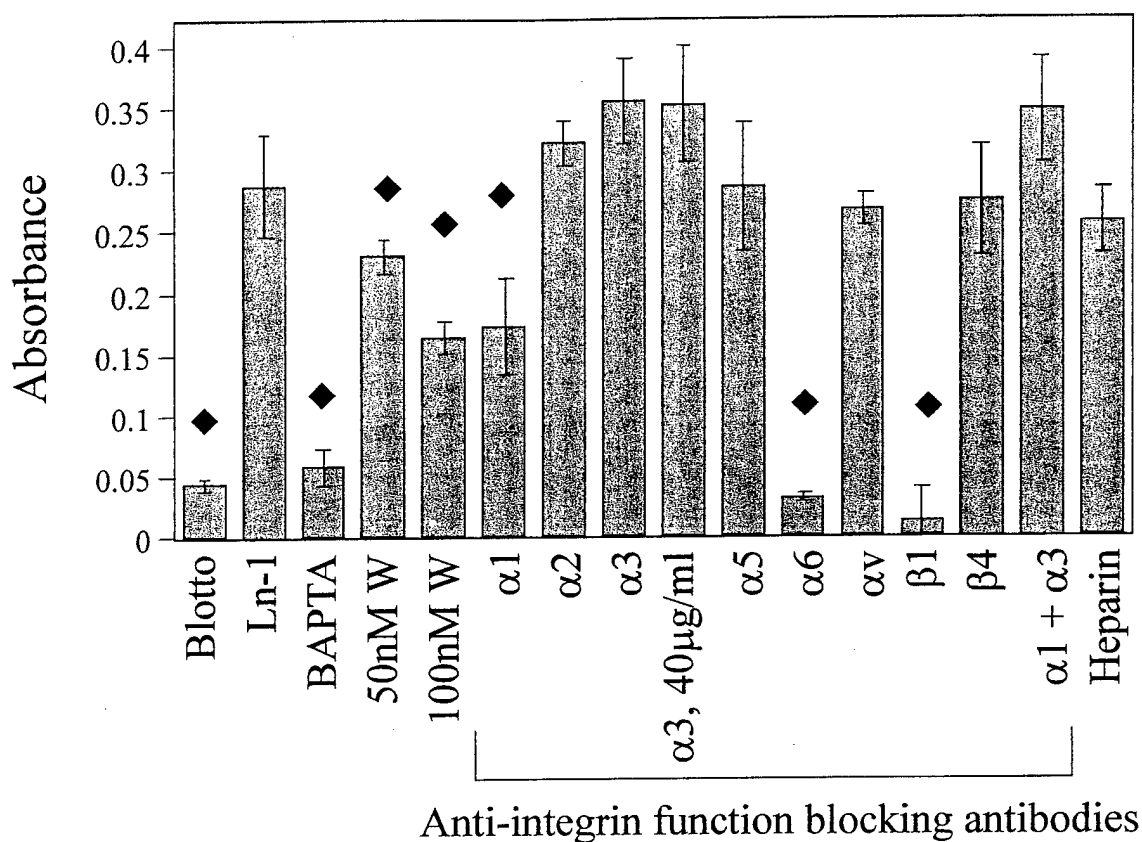


Figure 1. Properties of MCF-10A adhesion to EHS laminin-1. 120,000 cells per well were plated with the indicated compounds in wells coated with 20μg/ml laminin-1. Ln-1 = laminin-1 alone, BAPTA = BAPTA-AM, W = wortmannin. Error bars indicate standard deviation ($p=0.05$, $n=8$). Diamonds indicate a significant difference from uninfluenced adhesion to intact laminin-1.

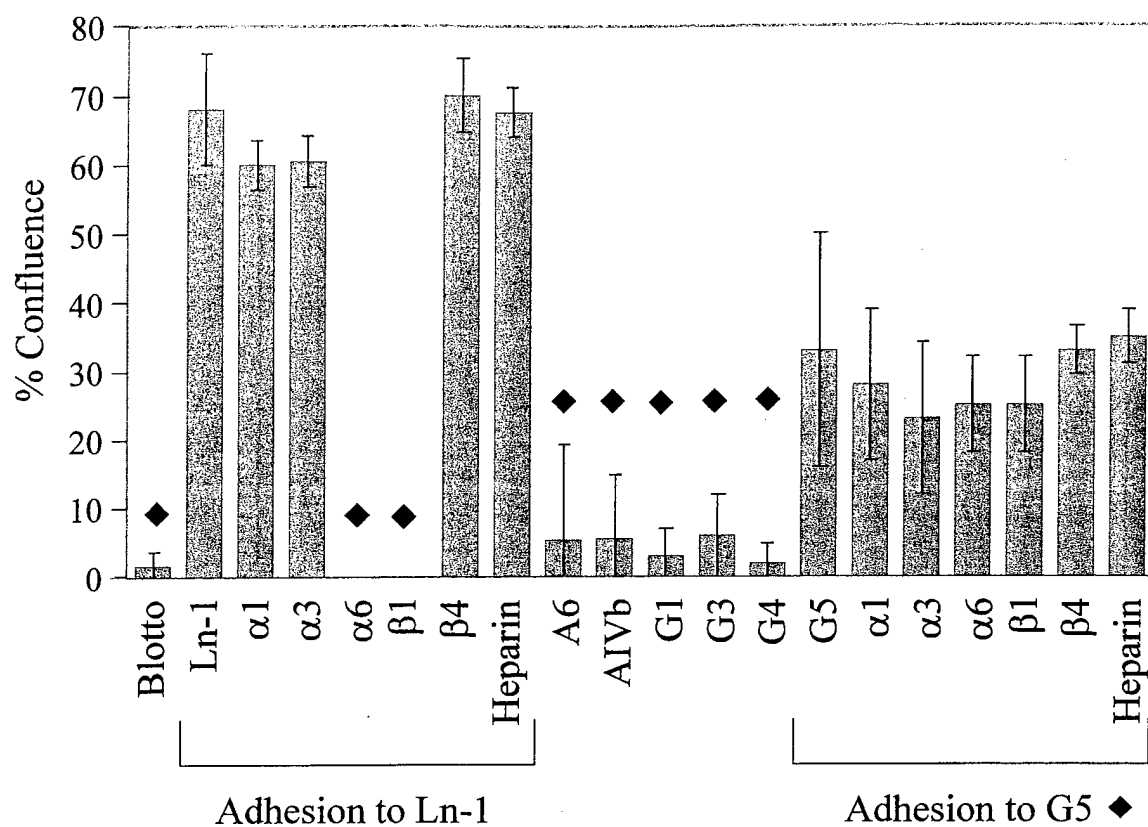


Figure 2. Properties of MDA-MB-231 cell adhesion to laminin-1 and protein G5. Assay conditions are identical to figure 3. Error bars indicate standard deviation ($p = 0.05$, $n=4$). Diamonds indicate significant difference from adhesion to laminin-1.

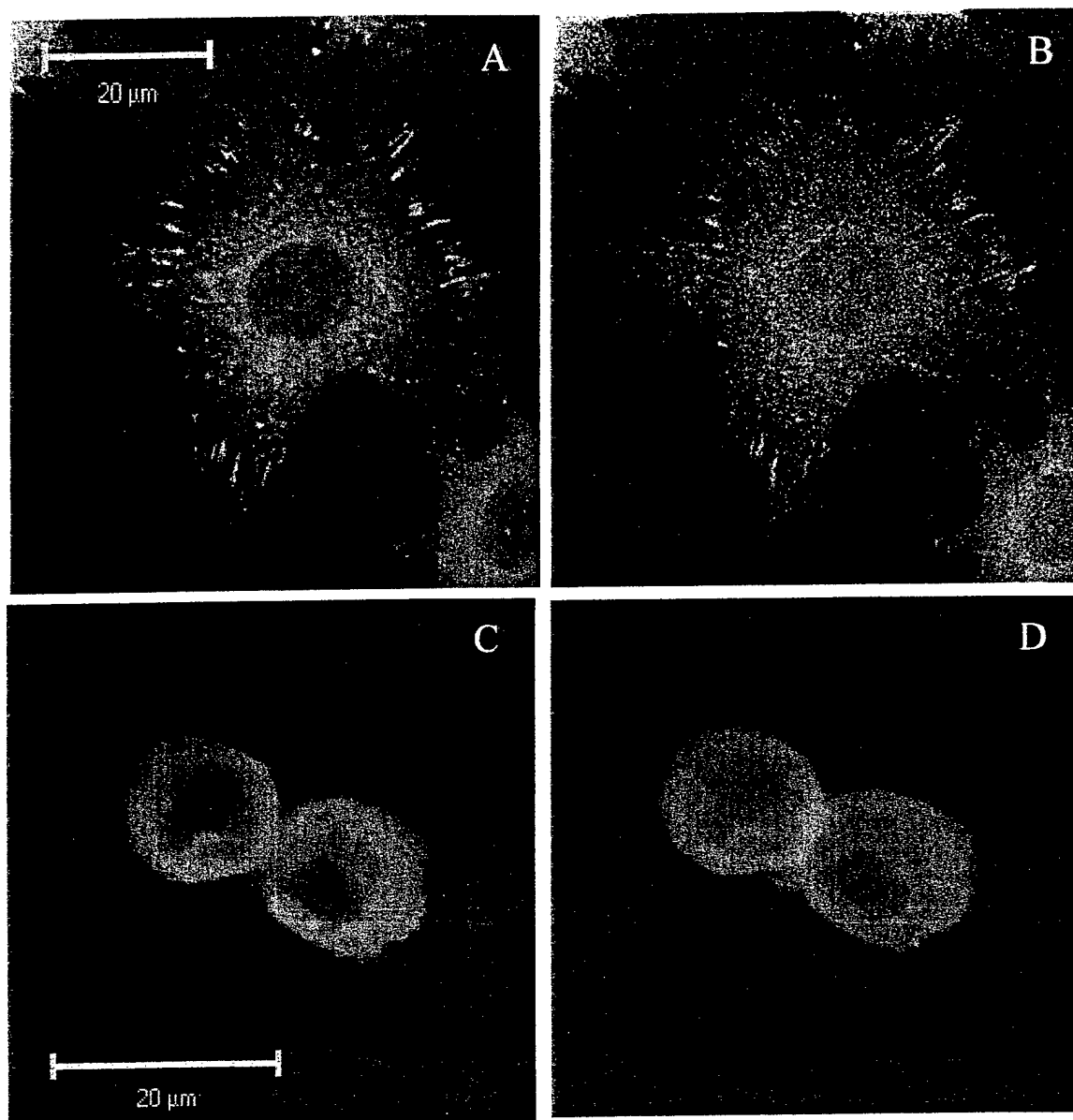


Figure 3. Morphology and focal complex formation of MDA-MB-231 cells adherent to laminin-1 and protein G5. Focal adhesions of cells adherent to laminin-1 (panel A) and protein G5 (panel C) were visualized by staining for the focal adhesion marker vinculin. Actin structure of cells adherent to laminin-1 (panel B) and protein G5 (panel D) were visualized by staining with the F-actin binding molecule phalloidin.

Antibody-Induced Activation of $\beta 1$ Integrin Receptors Stimulates cAMP-Dependent Migration of Breast Cells on Laminin-5

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The $\beta 1$ integrin-stimulating antibody TS2/16 induces cAMP-dependent migration of MCF-10A breast cells on the extracellular matrix protein laminin-5. TS2/16 stimulates a rise in intracellular cAMP within 20 min after plating. Pertussis toxin, which inhibits both antibody-induced migration and cAMP accumulation, targets the Gai3 subunit of heterotrimeric G proteins in these cells, suggesting that Gai3 may link integrin activation and migration via a cAMP signaling pathway. © 2000 Academic Press

Key Words: extracellular matrix; metastasis; signal transduction.

Laminins are a diverse group of heterotrimeric extracellular matrix proteins that constitute a major component of the basement membrane of epithelial tissues. The laminin-5 isoform, consisting of the $\alpha 3$, $\beta 3$, and $\gamma 2$ subunits, is abundantly expressed in the basement membrane of breast tissue [1] where it plays a role in mammary branching morphogenesis, and adhesion and migration of breast epithelial cells [2].

Evidence from both *in vitro* and *in vivo* studies support a functional role for laminin-5 in cell migration of both normal and malignant breast epithelial cells. Our laboratory has previously shown that *in vitro*, laminin-5 is the preferred adhesive substrate for breast epithelial cells [1]. In haptotactic migration assays, nontumorigenic breast cell lines fail to migrate significantly on laminin-5, whereas laminin-5 supports migration of highly malignant breast cell lines. *In vivo*, laminin-5 expression is enhanced in invading regions of metastatic breast tumors [3]. In addition, an altered conformation of laminin-5, resulting from proteolytic cleavage of the $\gamma 2$ chain by matrix metalloprotease 2, is

found at sites of tissue invasion, and this cleavage stimulates migration of otherwise nonmigratory breast cells *in vitro* [4]. Laminin-5 may contribute to the progression of tumorigenic breast cells from the stationary to malignant phenotype by stimulating enhanced migration of these cells.

Cells interact with laminins primarily through integrin receptors [5]. Ligand induced signal transduction by integrin/laminin binding regulates intracellular pH, tyrosine phosphorylation, inositol lipid metabolism, and calcium (Ca^{2+}) oscillations [6]. Signaling molecules known to associate with integrins receptors include protein tyrosine kinases, serine/threonine kinases, phospholipid kinases and lipases, ion channels, and members of the rho family of small molecular weight GTP binding proteins [6]. Laminin-5 is recognized by the $\alpha 3\beta 1$, $\alpha 6\beta 1$, and $\alpha 6\beta 4$ integrin receptors in a number of cell types, and the functional consequence of these interactions depend on the integrin receptor engaged. For example, ligation of laminin-5 with the $\alpha 6\beta 4$ integrin receptor supports branching morphogenesis and hemidesmosome formation in breast epithelial cells [2], while interaction with $\alpha 3\beta 1$ integrin supports migration of these same cells *in vitro* [7]. Little information is currently available on the specific signaling pathways triggered during these events.

While investigating the role of the $\alpha 3\beta 1$ integrin in motility of breast epithelial cells, we observed that haptotactic migration of the immortalized breast epithelial cell line MCF-10A on laminin-5 was stimulated by direct activation of the $\beta 1$ integrin receptor with the $\beta 1$ -activating monoclonal antibody TS2/16. Migration was dependent on intracellular cAMP signaling, and TS2/16-promoted a rise in intracellular cAMP levels that occurred 20 min after plating on laminin-5. Migration and cAMP accumulation were inhibited by treatment of the cells with pertussis toxin, a compound that inactivates the α subunit of the inhibitory class of heterotrimeric G proteins via ADP-ribosylation. We

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show that the G α i3 isoform is a target for ribosylation by pertussis toxin in these cells. Together these data present evidence that the β 1 integrin participates in the regulation of MCF-10A cell migration on laminin-5 through a cAMP-signaling pathway involving G α i3. This is the first description linking integrin activation to signaling through heterotrimeric G proteins.

MATERIALS AND METHODS

Cells

MCF-10A cells were maintained in DFCI medium according to Band and Sager [8]. MDA-MB-231 cells were cultured as described [1]. Rat 804G cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal calf serum and 1 \times Glutamine Pen-Strep solution (Irving Scientific). 804G cell conditioned medium was collected after 3 days of culturing and was clarified by centrifugation at 1500g.

Reagents

Mouse monoclonal antibodies against human integrin α 3 (clone PIB5) and β 1 (Clone P4ClO) were purchased from Gibco (Gaithersburg, MD). Mouse monoclonal antibody clone P5D2 against human β 1 integrin was purchased from Chemicon (Temecula, CA). Purified rat anti-mouse β 1 antibody 9EG7 was purchased from Pharmingen (San Diego, CA), and dialysed against PBS to remove sodium azide. Mouse monoclonal anti-human, activating β 1 integrin antibody TS2/16 (in ascites form) was generously provided by Dr. Martin Hemler (Dana Farber Cancer Institute, Boston, MA). Anti-rat laminin-5 monoclonal antibody TR1 was produced in this laboratory [9]. Both TS2/16 and TR1 were purified with a protein G affinity chromatography kit (Pierce, Rockland, IL). SQ22536 was purchased from Biomol (Plymouth Meeting, PA) and pertussis toxin from List Biological Laboratories, Inc. (Campbell, CA). 8-Bromo-cAMP, dibutyl cAMP, H-89, and forskolin were purchased from Calbiochem (San Diego, CA).

Adhesion and Migration Assays

Adhesion and migration assays were performed as previously described [1]. For anti-integrin antibody blocking experiments, antibodies were incubated with cells for 30 min before adding to assay wells, and were present throughout the assays.

cAMP Determination

Cells were collected by brief trypsinization, blocked with trypsin inhibitor, washed in DMEM, counted, resuspended at 1 \times 10⁶ cells/ml, and incubated at 37°C in migration medium/1 mM isobutylmethylxanthine (Sigma) to block phosphodiesterase activity. After 30

min, anti-integrin antibodies (TS2/16 or P5D2) were added, and cells were incubated at 37°C for an additional hour. Control cells were suspended in DMEM/1 mM isobutylmethylxanthine alone during this time. Cells (1 \times 10⁶/plate) were then plated on 35-mm dishes coated with affinity-captured laminin-5 [1] and incubated at 37°C for the indicated times. Cells representing the 0 time point were immediately retrieved from the dishes, collected by centrifugation, and lysed in cold cAMP extraction solution (95% ethanol, 5% 0.1 N HCl). After 10, 20, 30, and 90 min nonadherent cells were aspirated, plates were washed with PBS, and cAMP extraction buffer was added to the adherent cells. The PBS washes from each plate were centrifuged to collect loosely adherent cells, and these were added back to the appropriate extraction. All samples were kept on ice in cAMP extraction buffer for 2 h, then centrifuged to pellet precipitated protein. Protein was dissolved in 0.1 N NaOH and concentrations were determined with the BCA microassay (Pierce). Supernatants were evaporated and cAMP measured using a cAMP EIA kit (Perseptive Diagnostics, Inc., Cambridge, MA) as directed by the manufacturer. cAMP amounts were normalized to total protein in each sample and expressed as fmol/ μ g protein.

ADP Ribosylation Assay

Membrane preparation. Membranes were isolated from MCF-10A cells by lysis in ice-cold 10 mM Hepes pH 7.5, 3 mM MgCl₂, 2 mM EDTA containing 10 μ g/ml leupeptin, 2 μ g/ml aprotinin, and 0.5 mg/ml Pefabloc SC (Boehringer Mannheim Biochemicals, Indianapolis, IN). Cells were scraped, centrifuged to pellet nuclei, and the supernatant was collected. Membranes were pelleted from supernatant by centrifugation at 13,000g for 30 min at 4°C; and the pellets were resuspended in lysis buffer. Protein concentrations were determined by BCA protein assay (Pierce).

ADP-ribosylation and immunoprecipitation. ADP-ribosylation reactions were performed as described [10]. Final reaction conditions were as follows: 100 μ g membrane protein was suspended in 20 mM thymidine, 1 mM ATP, 1 mM GTP, 1 mM EDTA, 20 mM Hepes, pH 7.5 with or without 7.5 μ g pertussis toxin (activated prior to experiment by incubation for 10 min at 37°C in 20 mM DTT, 20 mM Hepes, pH 7.5) and 25 μ Ci ³²P-NAD (Specific activity = 30 Ci/mM, New England Nuclear catalog #BLU023). Reactions proceeded for 45 min at 30°C and were stopped by chilling to 4°C followed by a wash with 20 mM Hepes pH 7.5, 1 mM EDTA and 1 mM DTT. For SDS-PAGE analysis, membranes were solubilized in 50 μ l Laemmli sample buffer (LSB), heated for 5 min at 100°C and separated on a 12% SDS-polyacrylamide gel. ³²P-labeled proteins were detected by autoradiography of dried gels using Kodak X-Omat AR film with intensifying screens. For

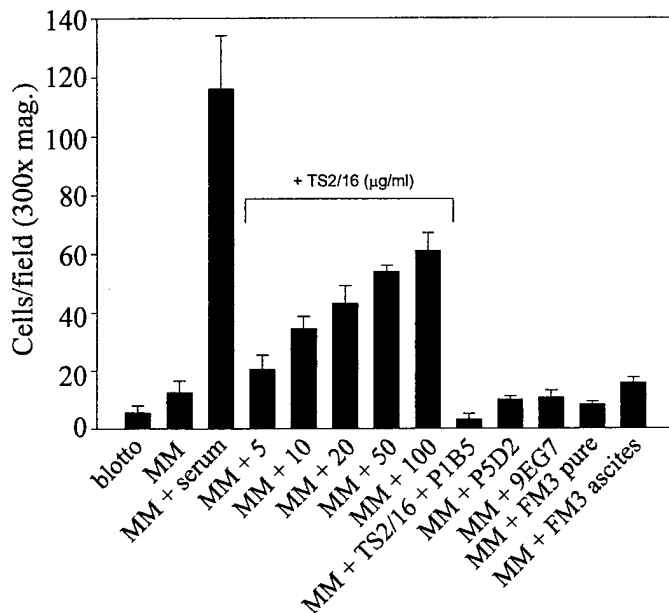


FIG. 1. The integrin activating antibody TS2/16 stimulates migration of MCF-10A breast cells on laminin-5. Indicated concentrations of TS2/16 were added to MCF-10A cells in a minimal medium lacking serum or other growth factors (MM) 15 min prior to plating in laminin-5 migration assays, and migrated cells were counted 18 h later. As controls, cells were plated in the presence of 10% serum, irrelevant mouse ascites (FM3 ascites), antibody purified from irrelevant ascites (FM3 pure), or nonfat dried milk (blotto). Results are expressed as the mean of eight measurements on two filters using 300 \times magnification, \pm standard deviation.

immunoprecipitations, ribosylated membrane proteins were solubilized in RIPA buffer containing protease inhibitors and were incubated with the following G-protein α subunit-specific peptide antibodies: I-20, specific for G α i1; C-10, specific for G α i3 (Santa Cruz Biotechnology). Immune complexes were captured by incubation with A/G agarose (Santa Cruz Biotechnology), solubilized by boiling in LSB and analyzed by SDS-PAGE as described.

RESULTS AND DISCUSSION

The β 1 Integrin-Activating Antibody TS2/16 Stimulated MCF-10A Migration on Laminin-5

The nontumorigenic breast cell MCF-10A remains statically adherent to laminin-5 via the α 3 β 1 integrin [1]. In haptotactic Transwell filter migration assays, these cells demonstrated only modest migration towards laminin-5. When preincubated with TS2/16, however, MCF-10A cells increased their migration in a dose-dependent manner towards laminin-5 (Fig. 1). TS2/16-treated cells also exhibited increased adhesion to laminin-5 (Fig. 2). These effects are not observed with other β 1 targeting antibodies (P5D2, 9EG7, Fig. 1; 9EG7, Fig. 2) or with TS2/16 on other substrates (data not shown). TS2/16 therefore stimulated a signaling

pathway that, concurrent with laminin-5 binding, led to enhanced cell migration. This pathway is dependent upon binding of the α 3 β 1 integrin, as pretreatment of the cells with the α 3 integrin-blocking antibody P1B5 completely blocked TS2/16-stimulated migration on laminin-5 (Fig. 1).

In each experiment, maximum stimulation of cell migration was observed when cells were allowed to migrate towards a gradient of fetal calf serum. This control was included in each migration assay to indicate the dynamic range of migration response in each population of cells. It is likely that this chemotactic migration was stimulated by the growth factors present in fetal calf serum, as serum-induced migration was inhibited by greater than 80% when cells were preincubated with antibodies that block the function of the epidermal growth factor receptor or drugs that inhibit tyrosine kinases (G. E. Plopper, unpublished data). It is therefore likely that serum was a stronger promigratory stimulant than TS2/16 because it activated several signaling pathways stimulated by soluble growth factors, while TS2/16 targeted integrin-associated signaling pathways.

The strength of cell adhesion to extracellular matrix ligands varies over a wide range and is under the control of both intracellular and extracellular cues. Work by Lauffenburger [11] suggests that very tight or very loose cell adhesion to matrix proteins will not support cell migration, and that migration occurs only when a medium-strength of adhesion is achieved. Thus, varying the potency of adhesion of integrin receptors for their ligands may be a critical step for

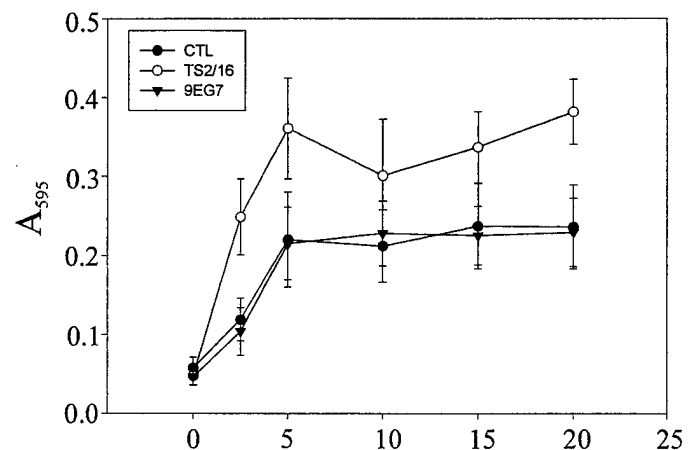


FIG. 2. TS2/16 antibody increases adhesion of MCF-10A cells to laminin-5. Cells were incubated in MM with 50 μ g/ml of TS2/16 or 9EG7 antibodies for 15 min, then were plated on affinity-captured laminin-5 for 30 min and adhesion quantified by measuring absorbance of crystal violet-dyed cells at 595 nm. Affinity capture was accomplished by successive addition of indicated concentrations of TR1 antibody, blotto, and 804G-conditioned medium containing soluble laminin-5. As a control (CTL), cells were incubated with no antibodies prior to plating. Results expressed as statistical mean \pm standard deviation ($n = 8$).

regulating cell migration. It is possible that TS2/16 stimulated migration in these cells by changing the strength of adhesion between $\alpha 3 \beta 1$ integrin and laminin-5, either directly or via activation of internal signaling pathways.

Alternatively, it is plausible that TS2/16 induced a conformational change in the $\beta 1$ integrin that mimicked binding to a promigratory form of laminin-5, such as those created through proteolytic processing. For example, cleavage of the $\gamma 2$ subunit of laminin-5 creates a conformation on which MCF-10A cells migrate constitutively [4, 12]. A promigratory laminin-5 can be converted to one that inhibits cell migration through cleavage of the $\alpha 3$ chain [13]. In both instances it is assumed that proteolytic processing masks or unmasks a promigratory domain on the intact laminin-5 trimer. This theory is also supported by studies showing that integrin activation by TS2/16 will rescue the growth of MCF-10A cells inhibited by treatment with laminin-5 blocking antibodies [14].

MCF-10A Cell Migration on Laminin-5 Is Modulated by cAMP

To define the mechanisms by which TS2/16 stimulated MCF-10A cell migration on laminin-5, we added inhibitors of known signaling molecules to antibody-stimulated cells in haptotaxis migration assays. We found that SQ22536, an inhibitor of adenylate cyclase,

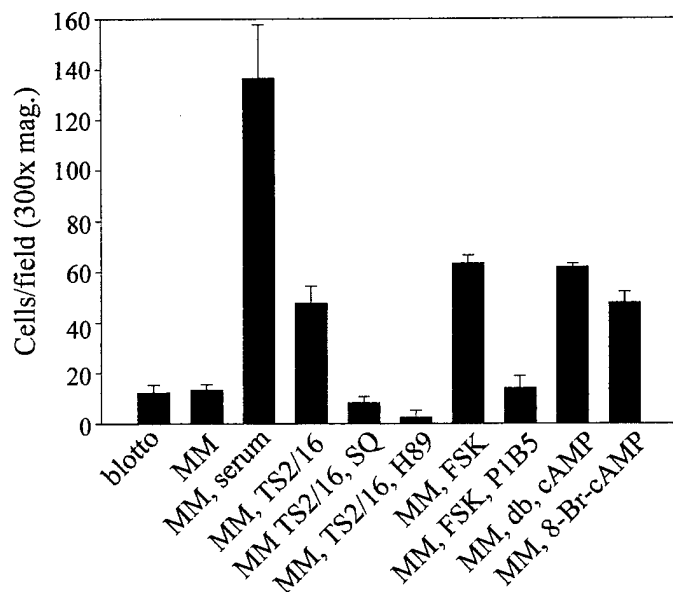


FIG. 3. Enhanced cAMP levels induce migration of MCF-10A cells on laminin-5. Cells were incubated in MM supplemented with 50 μ g/ml TS2/16, 50 μ g/ml P1B5, 250 mM SQ22536 (SQ), 4 μ M H89, 5 nM forskolin (FSK), 500 μ M dibutyryl cAMP (db cAMP), or 500 μ M 8-bromo-cAMP (8-Br-cAMP) for 15 min prior to adding to laminin-5 migration assays. As a control, cells were plated in the presence of serum or in MM on filters lacking laminins (blotto). Results are expressed as in Fig. 1.

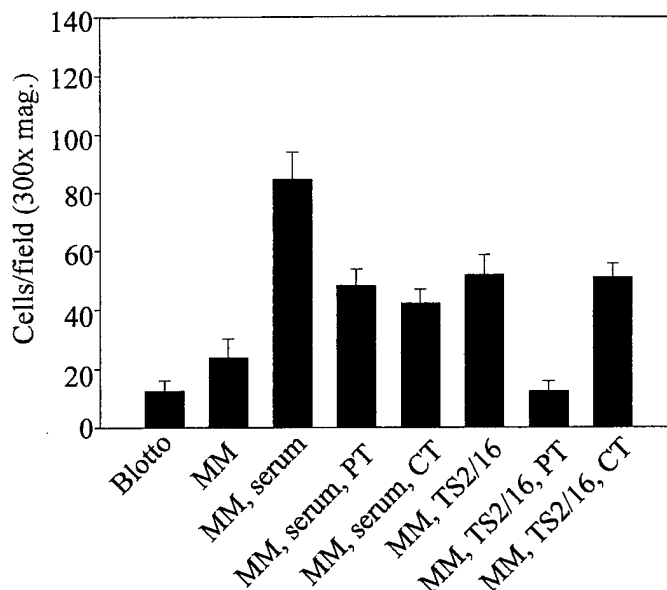


FIG. 4. Pertussis toxin inhibits TS2/16-stimulated migration on laminin-5. MCF-10A cells were suspended for 30 min in MM supplemented with either 10% serum or 50 μ g/ml TS2/16. 100 ng/ml pertussis toxin (PT), 100 ng/ml cholera toxin (CT), were added 15 min prior to plating cells in laminin-5 migration assays. As a control, cells suspended in MM were added to filters coated with blotto alone. Results expressed as in Fig. 1.

and H-89, an inhibitor of cAMP dependent protein kinase, completely blocked TS2/16 stimulated migration on laminin-5 (Fig. 3). In addition, pharmacological enhancement of cAMP levels with either forskolin or the nonhydrolyzable cAMP analogs 8-bromo-cAMP and dibutyryl cAMP were sufficient to enhance migration of MCF-10A cells on laminin-5 to levels stimulated by TS2/16 (Fig. 3). Prolonged (18 h) exposure to pertussis toxin, a compound that inhibits the cAMP signaling pathway mediated by the G α i class of signaling proteins, abolished migration on laminin-5 (Fig. 4) and reduced cAMP levels in MCF-10A cells over the same time course (Fig. 5B). These data established that cAMP was required for enhanced migration of MCF-10A cells on laminin-5.

Because adenylate cyclase activity is governed by different classes of heterotrimeric G proteins we exposed MCF-10A cells to pertussis toxin (an inhibitor of the G α i class) and cholera toxin (an inhibitor of the G α s class). While both pertussis and cholera toxin partially blocked serum stimulated migration of MCF-10A cells (approximately 50%), only pertussis toxin blocked TS2/16 stimulated migration on laminin-5 (Fig. 4). These data demonstrated that the specific pathway triggered by TS2/16 and laminin-5 was susceptible to regulation by G α i rather than G α s proteins, and again suggest that serum-stimulated migration resulted from activation of multiple signaling pathways, some of which utilize cAMP as a second messenger.

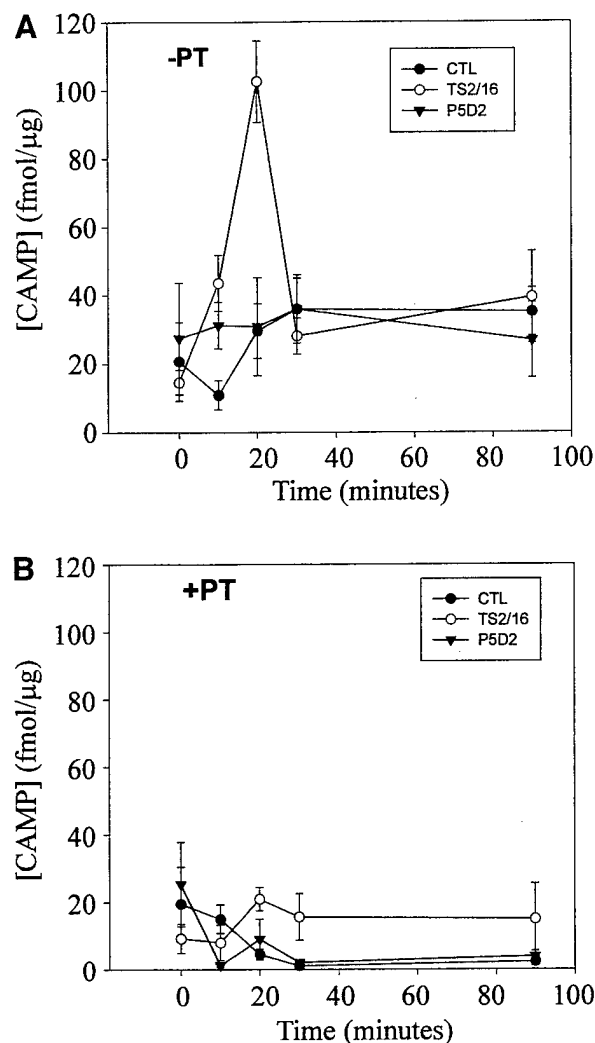


FIG. 5. Pertussis toxin inhibits a cAMP peak in TS2/16-stimulated cells. MCF-10A cells were suspended in MM supplemented with 20 $\mu\text{g/ml}$ TS2/16 or P5D2 antibodies and plated on laminin-5 for the indicated time, then lysed and assayed for total cAMP content by ELISA assay. As a control, cells were plated in the absence of antibodies (CTL). The experiments were performed (A) in the absence (-PT) or (B) presence (+PT) of 100 ng/ml pertussis toxin. Results are normalized to total cell protein for each time point and represent the mean of triplicate measurements for four experiments, \pm the standard error of the means.

Not all compounds that induced a rise in cAMP stimulated migration on laminin-5. Cholera toxin induced a transient rise in cAMP in MCF-10A cells (G. E. Plopper, unpublished observations), and stimulated growth of these cells when used in small quantities (ng/ml) in the low-serum media for these cells (DFCI medium) [8]. It is generally thought that small quantities of cholera toxin stimulate growth in these cells by activating signaling pathways used by G protein-linked chemokines (Gary Bokoch, Department of Immunology, The Scripps Research Institute, personal communication). Higher concentrations of cAMP ($\mu\text{g/ml}$) typically used to irreversibly activate Gas did not

stimulate haptotactic migration of MCF-10A cells on laminin-5 (G. E. Plopper, unpublished observations), suggesting that while cholera toxin does affect signaling in these cells, the pathways it affects do not play a role in integrin activated migration on laminin-5.

Chemotactic migration of many cell types is inhibitable by cholera and pertussis toxins [15, 16]. While pertussis toxin allows for unchecked cAMP production in the short term, prolonged pertussis toxin exposure suppressed cAMP levels in our cells, likely because of long-term desensitization of this pathway [17]. Although O'Connor *et al.* [18] reported that $\alpha 6 \beta 4$ expression suppressed cAMP levels in migrating breast cancer cells, no evidence has been published linking cholera and pertussis-sensitive signaling pathways to integrin-activated signaling.

These findings are consistent with our observation that numerous chemokines that modulate cAMP through Gas (bombesin, bradykinin, adrenaline) raised cAMP levels but failed to stimulate migration in our cells (G. E. Plopper, unpublished data). Each of these compounds exerts very distinct responses in breast cells, suggesting that while they share cAMP as a second messenger, they must generate specificity elsewhere in their signaling pathways. The specificity necessary to modulate haptotactic migration may be generated by localizing cAMP bursts to specific times and/or locations within a cell, by targeting specific isoforms of adenylate cyclase, or by integrating cAMP bursts with other integrin-associated behaviors (e.g., formation of focal adhesions, generation of cellular tension, activation of signaling pathways linked to migration in other cell types [e.g., those that utilize rho/ras G proteins or focal adhesion kinase]) [19].

Indeed, such integration appears to take place in smooth muscle cells, which exhibit increased migration on collagen upon activation of a cAMP signaling pathway linked to integrin associated protein and $\alpha 2 \beta 1$ integrin; this activation also stimulates the mitogen activated protein kinase ERK, and is inhibited by pertussis toxin. [20]. In this study, migration is stimulated upon a reduction in cAMP levels and is inhibited by analogs of cAMP. The differences between these findings and ours may be attributed to differences in cell type, migratory stimulus, migratory substrate, and/or integrin receptor involved: we have observed that inhibition of $\alpha 2 \beta 1$ integrin stimulates haptotactic $\alpha 3 \beta 1$ -mediated migration in our cells, for example (G. E. Plopper, unpublished findings).

TS2/16 Stimulated a Rise in Intracellular cAMP via a Pertussis Toxin-Sensitive Signaling Pathway

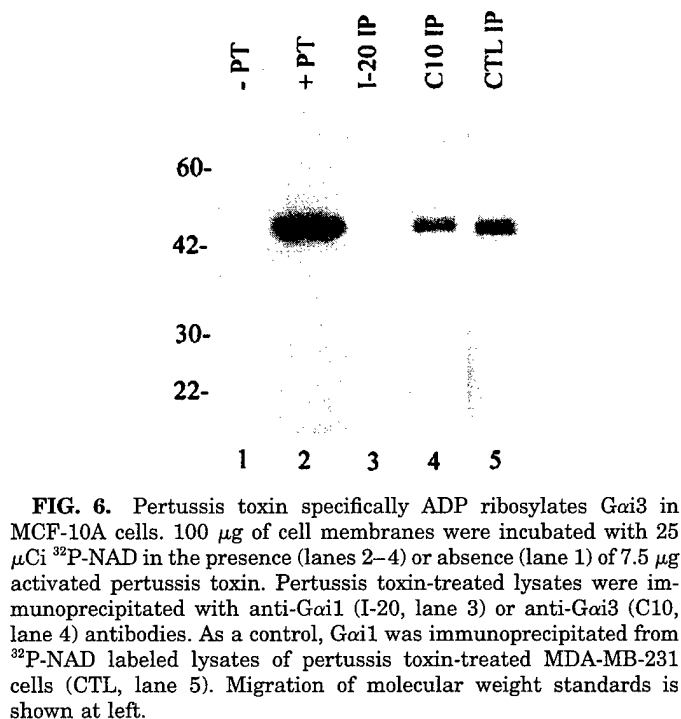
Since pertussis toxin alters intracellular cAMP levels, and cAMP modulation was sufficient to enhance migration in our cells, we examined the levels of cAMP in TS2/16-stimulated cells plated on

laminin-5. Within 20 min after plating, cAMP levels were raised approximately fourfold in TS2/16 treated cells. This peak occurred within the time frame of integrin signaling [6]. Enhanced cAMP accumulation was specific to TS2/16, and not a product of integrin clustering, as neither cells treated with the nonactivating $\beta 1$ antibody P5D2 nor cells plated on laminin-5 without antibodies exhibited enhanced cAMP production (Fig. 5A). Preincubation with pertussis toxin completely eliminated this peak but did not significantly affect basal cAMP levels (Fig. 5B). Concurrent stimulation by laminin-5 adhesion and TS2/16 are required, as cAMP levels did not change in suspended cells treated with TS2/16 (G. E. Plopper, unpublished). It appeared, therefore, that the combination of intact laminin-5 and TS2/16 pretreatment stimulated a signaling pathway involving cAMP that was specifically blocked by pertussis toxin.

Pertussis Toxin ADP-Ribosylated Gai3 in MCF-10A Cells

Pertussis toxin ADP ribosylates the Gai class of heterotrimeric G proteins. To determine the repertoire of Gai subunits expressed in MCF-10As we performed Western blot analysis of whole cell lysates and isolated membrane fractions using polyclonal antibodies raised against specific G protein subunits. These studies revealed that MCF-10A cells expressed Gai1 and Gai3, but not Gai2 (data not shown). To establish the targets of pertussis toxin in these cells we carried out ADP-ribosylation assays in the presence of ^{32}P -NAD. Addition of pertussis toxin specifically induced the ribosylation of a 43-kDa protein (Fig. 6, lane 2). No ^{32}P labeled proteins are detectable without addition of pertussis toxin (Fig. 6, lane 1). The molecular weight of the ribosylated protein was consistent with that of the α subunits of heterotrimeric G proteins. The identity of this protein was determined by immunoprecipitation of the ribosylated membrane proteins with Gai1 and Gai3 antibodies. Antibody C10, which reacts primarily with Gai3, immunoprecipitated a band of 43 kDa (Fig. 6, lane 4). The anti-Gai1 antibody I-20 failed to precipitate any ADP ribosylated proteins in MCF-10A cells (Fig. 6, lane 3), but did precipitate a 43-kDa band from a control cell line, MDA-MB-231 (Fig. 6, lane 5). Therefore, pertussis toxin ribosylated Gai3, but not Gai1 in MCF-10A cells.

In addition to controlling adenylate cyclase activity, Gai3 is associated with and activates amiloride-sensitive Na^+ channels [21], which are expressed in many epithelial cells including breast. These channels are also regulated by the actin cytoskeleton [22] and cAMP dependent protein kinase [23], suggesting that Gai3 may link integrin-mediated actin polymerization, cAMP signaling, cAMP dependent protein kinase ac-



tivity, and amiloride-sensitive channel activation. Curiously, amiloride also suppresses lung metastases from breast tumors [24]; our data suggest that it may do so, at least in part, by inhibiting tumor cell migration.

In conclusion, we report that the $\beta 1$ integrin-stimulating antibody TS2/16 induced migration of MCF-10A cells on laminin-5 that was dependent upon cAMP linked signaling. TS2/16 also stimulated a rise in intracellular cyclic AMP within 20 min after plating on laminin-5. Both the enhanced migration and cAMP peak were inhibited by pertussis toxin. Pertussis toxin targeted the Gai3 subunit of heterotrimeric G proteins in these cells. This evidence suggests that the $\beta 1$ integrin participates in the control of MCF-10A cell migration on laminin-5 via a cAMP signal pathway regulated by Gai3. This form of signaling, beginning with an external stimulus of the integrin receptor, is referred to as "outside-in signaling" to differentiate it from changes in integrin function resulting from activation of internal signaling pathways [6]. We propose that TS2/16 mimics the effects of proteolytic processing of laminin-5 by forcing the $\alpha 3 \beta 1$ integrin into a conformation formed by binding promigratory forms of laminin-5. We are currently examining the effect of these proteolytic modifications on intracellular signaling activities in MCF-10A cells. Because acquisition of a migratory phenotype is required for malignant progression of tumorigenic breast cells, elucidating pathways involved in enhanced migration of breast may lead to discovery of novel targets for anticancer therapies.

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The WD protein Rack1 mediates protein kinase C and integrin-dependent cell migration

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SUMMARY

The scaffolding protein, Rack1, is a seven-WD-domain-containing protein that has been implicated in binding to integrin β subunit cytoplasmic domains and to members of two kinase families (src and protein kinase C, PKC) that mediate integrin bidirectional signaling. To explore the role of Rack1 in integrin function we have transfected this protein in Chinese hamster ovary (CHO) cells. We have observed no effect of Rack1 overexpression on inside-out signaling as the ligand binding properties of CHO cells also expressing constitutively active or inactive integrins were not affected. In contrast, we observed that cells stably or transiently overexpressing Rack1 had decreased migration compared to mock transfected cells. Stable Rack1 transfectants also demonstrated an increased number of

actin stress fibers and focal contacts. These effects on motility and cytoskeletal organization did not appear to result from Rack1 inhibition of src function as downstream substrates of this kinase were phosphorylated normally. In addition, expression of an active src construct did not reverse the migratory deficit induced by Rack1 overexpression. On the other hand when we overexpressed a Rack1 variant with alanine substitutions in the putative PKC binding site in its third WD domain, we observed no deficit in migration. Thus the ability of Rack1 to bind, localize and stabilize PKC isoforms is likely to be involved in aspects of integrin outside-in signaling.

Key words: Rack1, Migration, Integrin, Protein kinase C

INTRODUCTION

The orderly and controlled migration of cells is crucial in many aspects of physiology including embryonic development, immune function, wound repair and angiogenesis. Unregulated or abnormal cell motility also underscores certain pathological situations such as the invasion and metastasis of cancer cells. Cell migration is a complex response to external stimuli involving an interplay between cellular adhesive events and cytoskeletal organization. Signal transduction events involving the regulation of integrin affinity (Huttenlocher et al., 1996), small GTPases (Nobes and Hall, 1995; Nobes and Hall, 1999; Takaishi et al., 1993), the ras/MAP kinase pathway (Klemke et al., 1997; Nguyen et al., 1999), Cas/Crk coupling (Klemke et al., 1998), focal adhesion kinase (Cary et al., 1996; Ilic et al., 1995), phosphatidylinositol 3-kinase (Rodriguez-Vicana et al., 1997), PLC γ (Chen et al., 1994), protein kinase C (Laudanna et al., 1998; Ng et al., 1999; Rigot et al., 1998) and calpain (Huttenlocher et al., 1997), have all been implicated in regulating integrin-mediated adhesion, integrin downstream signaling or actin polymerization leading to motile behavior. Understanding the molecular events that regulate cell migration will be important in designing ways to control this event.

Rack1 is a 36 kDa cytosolic protein composed of seven WD motifs and thus is structurally similar to a G protein β subunit. It was originally identified as a receptor for activated protein kinase C (Ron et al., 1994). In this mode of action, Rack1 acts as neither a substrate nor an inhibitor, but rather enables the

translocation of PKC isozymes and stabilizes their active forms (Mochly-Rosen and Gordon, 1998). Indeed, reduced Rack1 levels are correlated with defective PKC translocation in the aging rat brain (Battaini et al., 1997) and disruption of PKC-Rack1 interactions impaired insulin-induced kinase translocation, *Xenopus* oocyte maturation (Ron et al., 1995) and regulation of calcium channels in cardiomyocytes (Zhang et al., 1997). Rack1 has also been isolated in a yeast two-hybrid screen using an src SH2 domain as bait (Chang et al., 1998). Additional studies suggested that the interaction of Rack1 with src family members inhibited the activity of these kinases in vitro and that Rack1 overexpression in NIH 3T3 and 293T cells decreased growth rates and levels of tyrosine-phosphorylated proteins, respectively (Chang et al., 1998). Thus, Rack1 appeared to have opposing effects on the kinases to which it binds: stabilizing the active conformation of PKC but inhibiting members of the src family. Additional binding partners of Rack1 include integrin β subunit cytoplasmic domains (Liliental and Chang, 1998; Zhang and Hemler, 1999), a phosphodiesterase isoform (Yarwood et al., 1999), certain pleckstrin homology (PH) domains (Rodriguez et al., 1999) and the common β chain of the IL-5/IL-3/GM-CSF receptor (Geijsen et al., 1999). While the functional significance of these latter associations remains undefined, a role for Rack1 as a scaffolding protein is suggested.

Since PKC and src family kinases have been implicated in integrin bidirectional signaling, we wished to explore the functional consequences of Rack1-integrin association. When transiently or stably overexpressed in CHO cell lines, Rack1

did not appear to affect ligand binding properties. However these transfectants did demonstrate decreased integrin-dependent cell migration and an increased number of actin stress fibers and focal contacts compared to wild-type cells. These effects did not appear to be due to inhibition of src kinase activity and were not reversed with an active src construct. In addition, these migratory defects were not observed when we utilized a Rack1 construct with a mutated PKC binding site. These results suggest that Rack1 is involved in integrin outside-in signaling in a manner that involves its interaction with PKCs but is independent of its reported effects on src.

MATERIALS AND METHODS

Reagents

A monoclonal antibody against Rack1 was obtained from Transduction Labs while one against talin was from Sigma. An $\alpha_{11b}\beta_3$ antibody (D-57) and the ligand mimetic monoclonal, Pac1, were obtained from Mark Ginsberg and Sandy Shattil, Scripps Research Institute, respectively. All other antibodies (HA epitope, crk, cas, ERK1 and ERK2, phospho-ERK and phosphotyrosine) were from Santa Cruz Biotechnologies. The conjugated reagents, phycoerythrin-streptavidin and FITC-IgG and IgM were from Molecular Probes and Biosource, respectively. The calcein AM dye and rhodamine-phalloidin were obtained from Molecular Probes while matrix proteins were from Enzyme Research Laboratories Inc. Restriction enzymes were from New England Biolabs or Boehringer Mannheim. All synthetic oligonucleotides were from Genosys.

cDNA cloning

A partial cDNA for Rack1 encoding residues 81-317 was isolated in a yeast two-hybrid screen using a HeLa cell library and the β_1 tail as a bait. Two-hybrid screening, yeast manipulations and liquid β -galactosidase assays were done as per the manufacturer's (Clontech) recommendations. The remaining coding and 5' non-coding sequences of Rack1 were isolated by RACE-PCR with HeLa Marathon-Ready cDNA (Clontech), subcloned into the TA cloning vector (Invitrogen) and confirmed by DNA sequencing. A full-length clone containing a C-terminal HA tag was then generated in the pCDNA3.1 vector (Invitrogen) by first amplifying the partial yeast clone with a 3' oligonucleotide encoding an HA-tag and an *Xba*I site and a 5' oligonucleotide complementary to the yeast plasmid pB42AD, followed by digestion of the product with *Syl*I and *Xba*I. Next, remaining Rack1 sequences in the TA clone were isolated by digestion with *Nor*I and *Syl*I and finally the two Rack1-encoding pieces ligated into *Nor*I and *Xba*I-digested pCDNA3.1. An inducible and myc-tagged expression clone for Rack1 was established by subcloning in the pINDHygro vector (Invitrogen). To generate alanine substitutions in the third and sixth WD domains of Rack1 we used appropriately designed oligonucleotides in a site-directed mutagenesis strategy (Quick Change Mutagenesis; Stratagene). All constructs were verified by sequencing before use.

Cell culture, transfections and analysis of signaling properties

All CHO cells were maintained in DMEM medium plus 10% fetal calf serum (FCS) and 1% glutamine, nonessential amino acids and penicillin/streptomycin. Stable Rack1 transfectants were generated in wild-type or $\alpha_{11b}\beta_3$ -expressing (A5) CHO cells (O'Toole et al., 1990) by cotransfection with HA-tagged Rack1 in pCDNA3.1 and pZeo (Invitrogen) using the lipofectamine reagent (Life Technologies). The medium was changed after 24 hours and after 48 hours the cells were grown in medium containing 250 μ g/ml zeocin (Invitrogen). After 2 weeks, colonies were picked, scaled up and analyzed for HA-Rack1

expression by western blotting with anti-Rack1 or anti-HA antibodies. The ligand-binding properties of these transfectants were determined by flow cytometric analysis of Pac1 binding (O'Toole et al., 1990).

Transient expression of Rack1 was achieved using a ponasterone inducible system (Invitrogen). Briefly, the appropriate cell types were cotransfected with the pHOOK vector (Invitrogen) to select for transfectants, pVgRxR and the pIND vector containing full-length Rack1. 24 hours after transfection the medium was changed to one containing 0.5% FCS and 5 μ M ponasterone (Invitrogen) and the cells were allowed to grow for another 20-24 hours. The transfectants were then isolated by magnetic sorting and induced Rack1 expression was determined by western blotting with an anti HA-antibody. Expression levels were determined by densitometry with an image analyzer (Alpha Imager 2000, Alpha Innotech Corporation).

To determine the effects of Rack1 overexpression on adhesion-stimulated signaling, we first incubated the appropriate cell types overnight in medium containing 0.5% FCS. The cells were then harvested with trypsin, washed in medium containing 0.5 mg/ml soybean trypsin inhibitor, resuspended in medium containing 0.2% BSA and incubated in suspension culture dishes for 2 hours at 37°C. At this time, some of the cells were collected and washed in phosphate-buffered saline (PBS), while the remainder were allowed to adhere to fibrinogen (fg)-coated dishes (15 μ g/ml) for various times. These adherent cells were then likewise collected and washed and all cells lysed on ice in a buffer consisting of 20 mM Tris-HCl, pH 7.4, 250 mM NaCl, 0.5% NP-40, 3 mM EGTA, 5 mM EDTA, 20 mM NaPi, 3 mM β -glycerophosphate, 1 mM Na_3VO_4 , 1 mM PMSF, 10 mM NaF and 1 \times complete protease inhibitor (Boehringer Mannheim). The lysates were clarified by centrifugation and protein concentration determined by BCA assay (Pierce). For immunoprecipitations, 300 μ g of lysate was incubated with the appropriate antibody overnight at 4°C and proteins recovered with protein G-sepharose. Isolated proteins were resolved by SDS-PAGE, transferred to nitrocellulose and detected after incubation with primary antibodies, horseradish-peroxidase (HRP)-conjugated secondary antibodies, and the ECL reagent (Amersham). For analysis of ERK1/2 activity levels, 60 μ g of crude lysate were directly used in western blotting analysis with phospho-ERK and ERK1/2-specific antibodies.

Migration assays

Transwells were prepared by coating the bottom surface of the membrane with matrix proteins (50 μ g/ml in carbonate buffer (0.2 M $\text{Na}_2\text{CO}_3/\text{NaHCO}_3$, pH 9.3)) for a minimum of 1 hour, and then blocked with 5% milk in PBS plus 0.2% Tween 20. The cells to be examined were incubated in medium containing 0.5% serum overnight and then harvested and resuspended in serum-free medium at approximately 10⁶/ml. 0.5 ml of serum-free medium was applied to the bottom chamber while 100 μ l of the resuspended cells were then applied to the top of the Transwell, and migration was allowed to proceed for 8-16 hours. At this time medium in the bottom well was replaced with some of the same containing 5 μ M calcein AM and migration allowed to proceed for a final 30 minutes. The Transwells were then washed 2 \times in PBS, the remaining cells on the top wiped off with a Q-Tip, and fluorescence read (excitation 485 nm, emission 530 nm) in a CytoFluor II. These values were normalized relative to the fluorescence of total cells initially seeded to the top of the Transwell and expressed as a percentage of control samples. The control samples were untransfected cells (in experiments where stable transfectants were used) or vector-transfected cells (in experiments where transient transfectants were used).

Immunofluorescence

Glass coverslips were coated with 10 μ g/ml fg in carbonate buffer for 1 hour at room temperature and then blocked in 1% BSA. 5 \times 10⁴ cells were then added and allowed to adhere for 2 hours at 37°C. At this time the cells were fixed with 1% paraformaldehyde, permeabilized

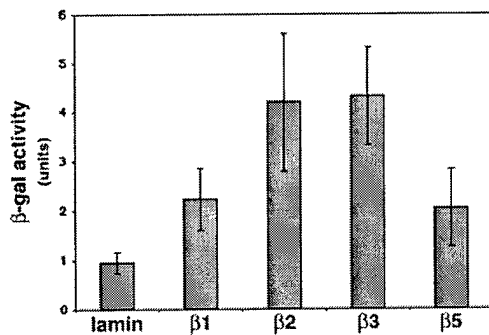


Fig. 1. Rack1 interacts with several integrin β subunit cytoplasmic domains. The interaction of full-length Rack1 with a non-specific clone (laminin) and with the cytoplasmic sequences of the β_1 , β_2 , β_3 and β_5 integrins was determined by yeast two-hybrid methodology and quantitated in a β -galactosidase assay (arbitrary units). In addition to previously reported interactions with β_1 , β_2 and β_5 , Rack1 also interacts with β_3 .

with 0.1% Triton and incubated successively with a monoclonal anti-talin antibody, FITC-conjugated goat antimouse, rhodamine-phalloidin and Toto-3. The coverslips were then gently washed, mounted on glass slides and visualized using a fluorescence microscope.

RESULTS

Inside-out signaling is not affected by Rack1 overexpression

Rack1, a receptor for activated protein kinase C, has been implicated in binding to the integrin β_1 , β_2 and β_5 cytoplasmic domains using the yeast two-hybrid approach (Liliental and Chang, 1998; Zhang and Hemler, 1999). Our two-hybrid studies confirm these results and also suggested an interaction with the β_3 cytoplasmic domain (Fig. 1). In addition, Rack1 has been implicated in binding to both protein kinase C and src

family members, two mediators of integrin bidirectional signaling (Chang et al., 1998; Ron et al., 1994). To begin to examine the role of Rack1 in integrin-mediated functions we stably overexpressed an HA-tagged form of this protein in CHO cells, which also express recombinant $\alpha_{IIb}\beta_3$ (A5 cells). After G418 selection and western blotting analysis of individual clones, we identified and maintained two clonal lines for further study. These two lines (R8 and R47) demonstrate both the slower migrating HA-tagged Rack1 (upper bands) as well as the endogenous Rack1 (lower bands) (Fig. 2A). Scanning the western blots suggested a 1.5- to twofold increase in Rack1 expression over levels in untransfected cells. Expression of HA-Rack1 in the A5 cells did not alter integrin expression levels (not shown) or gross morphological features. In all of the subsequent functional analyses described below, the behavior of the two clonal lines appeared identical.

As activators of protein kinase C have been widely used to stimulate integrin binding function, we first sought to determine whether Rack1 overexpression might affect integrin affinity state. Using the monoclonal antibody Pac1 as a ligand mimetic, however, we demonstrated that the clonal lines R8 and R47 remain in the same low-affinity state as their parental line, A5 (Fig. 2B). Ligand binding in these cells could only be stimulated with an activating antibody. Furthermore, Rack1 does not suppress ligand binding when overexpressed in cells with constitutively active, high-affinity integrins (data not shown). Thus Rack1 overexpression does not appear to affect inside-out signaling.

Rack1 overexpression alters cytoskeletal structure, focal contact formation and cell migration

To begin to explore the effects of Rack1 on outside-in signaling, we first examined the cytoskeletal organization of the Rack1 overexpressing clones upon adhesion to fg. Immunofluorescence staining of wild-type CHO cells and the clonal line R8 with an anti-talin antibody and phalloidin demonstrated distinct differences between the two cell types. Specifically, the Rack1 overexpressers demonstrated a

Fig. 2. The overexpression of Rack1 does not affect integrin inside-out signaling. (A) To explore its effects on integrin binding affinity, we stably expressed Rack1 in a CHO line also expressing $\alpha_{IIb}\beta_3$ (A5 cells). Two clonal lines (R8 and R47) expressing both wild-type Rack1 (lower band) and the HA-tagged and transfected Rack1 (upper band) were identified and carried on for further study. (B) The binding of the ligand mimetic Pac1 to A5 cells and the two clonal lines was determined in a flow cytometry assay. Rack1 overexpression did not stimulate high levels of Pac1 binding (solid line) and this could only be accomplished after stimulation of the cell types with an activating antibody, LIBS 6 (shaded histogram). Excess RGD-containing peptide was used in these assays to block binding and demonstrate specificity (dotted line).

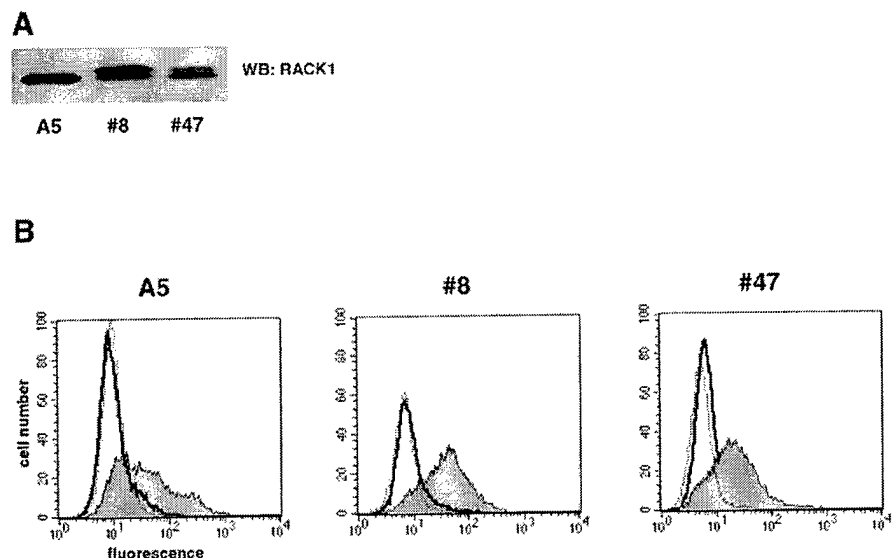
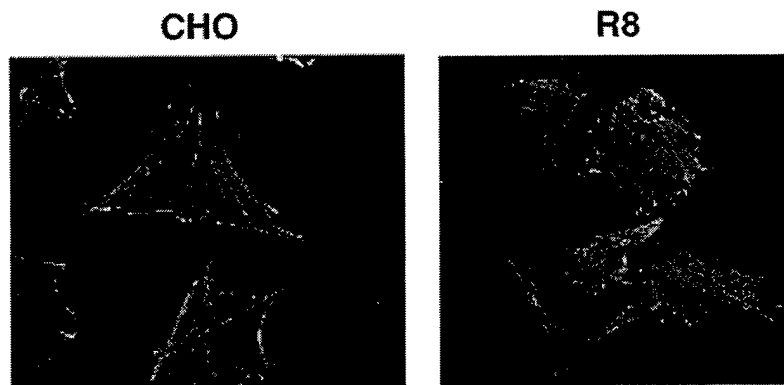


Fig. 3. Rack1 overexpression alters cytoskeletal organization. Parental CHO cells (left) and the Rack1 overexpressing clone R8 (right) were allowed to adhere to fg and then subjected to immunofluorescence staining as described in Materials and Methods. Analysis was with rhodamine-phalloidin (red) to visualize actin stress fibers and a talin antibody (green) to visualize focal contacts. Rack1 overexpressing cells demonstrate an increase in stress fibers and focal contacts when compared to parental cells.



noticeable increase in both the number of stress fibers and focal contacts relative to wild-type cells (Fig. 3). Thus Rack1 overexpression affected the cytoskeletal organization of adherent cells.

The cytoskeletal organization of the adherent, Rack1-overexpressing cells was similar to the observed phenotype of FAK knockout cells (Ilic et al., 1995). Since these cells also demonstrate migration defects, we next sought to determine what effect Rack1 might have on integrin-mediated migration. Using a Transwell migration assay, we have consistently observed that the clonal line R8 demonstrated a 30-60% decrease in migration on fg-coated wells when compared to the parental cell line, A5 (Fig. 4A). As these studies were done with clonal lines, we also wished to see if transient overexpression of Rack1 would have a similar effect on migration. To do this, we subcloned HA-tagged Rack1 into the pINDHYGRO vector (Invitrogen) and induced its expression with the ecdysone analog, ponasterone (Fig. 4A). These transient transfectants reproducibly demonstrated an approximately twofold increase in Rack1 expression. Similar to those results with the stable lines, transient overexpression of Rack1 in A5 cells also deterred migration on fg (Fig. 4A). Next, we wished to determine if these Rack1 effects were specific to these CHO, $\alpha_{11b}\beta_3$ -expressing cells. We therefore stably overexpressed Rack1 in wild-type CHO cells (clonal line CR22) and examined their migratory behavior on fn, which was mediated by endogenous $\alpha_5\beta_1$ (Fig. 4B). As with

the A5 cells, overexpression of Rack1 in CHO cells inhibited cell migration. Finally, we observed similar effects on migration when we performed these CHO cells studies in a transient manner (Fig. 4B). Thus migration mediated by different integrins was inhibited.

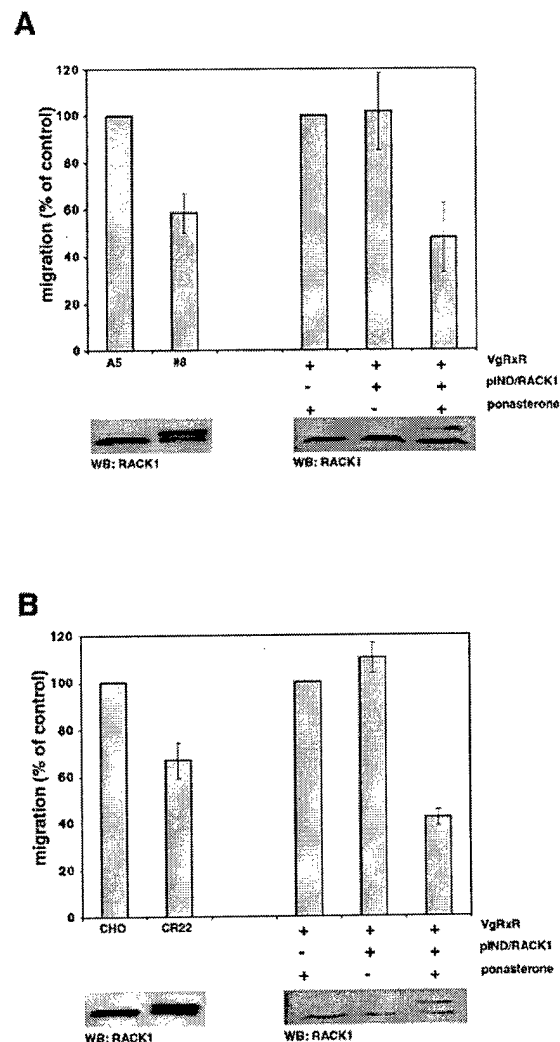


Fig. 4. Rack1 overexpression inhibits cell migration. (A) A5 cells and the clonal line R8 were allowed to migrate on fg-coated Transwells and the number of migrated cells quantitated by calcein AM staining as described in Materials and Methods (left bars). The stable Rack1 overexpressing line demonstrated an approximate 60% decrease in cell migration relative to the parental line. A5 cells were also transiently transfected with pHOOK and variably with pVgRxR and pINDRack1 as indicated (right bars). Protein expression was induced after 24 hours with 5 μ M ponasterone and transfectants isolated after 48 hours by magnetic sorting. Expression of Rack1 was determined by western blotting with an anti-Rack1 antibody (bottom) while migratory properties were analyzed as above in a Transwell assay. (B) Stable expression of Rack1 was also accomplished in wild-type CHO cells. Migration of the parental cells and a Rack1 overexpressing clone (CR22) on fn-coated Transwells were examined as above (left bars). Finally, transient Rack1 expression and migration was accomplished in CHO cells as described above (right bars and bottom). In all cases overexpression of Rack1 inhibited cell migration relative to parental cells.

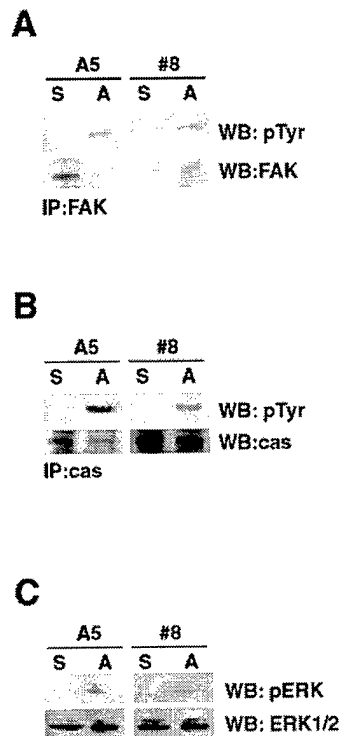


Fig. 5. Rack1 does not affect downstream signaling from src. Parental cells (A5) or the Rack1 overexpressers (R8) were serum starved overnight and then kept in suspension for 2 hours. At this time some of the cells were harvested and lysed (S) while the remainder were allowed to adhere to fg-coated dishes (A) for 10 minutes and then harvested and lysed. (A) Equal amounts of lysate were then immunoprecipitated with an anti-FAK antibody and isolated proteins resolved by SDS-PAGE and subjected to western blotting with anti-phosphotyrosine and anti-FAK antibodies, as indicated. Lysates prepared as described above were also immunoprecipitated with an anti-cas antibody and analyzed by western blotting with anti-phosphotyrosine and anti-cas antibodies (B). Finally 60 μ g of lysate from suspension and adherent cells were resolved and analyzed by western blotting with a phospho-ERK specific antibody (C). The same blot was stripped and then reprobed with antibodies to ERK1 and ERK2. There appeared to be no deficiencies in FAK phosphorylation, cas phosphorylation, and ERK1/2 activation when we compared parental and Rack1 overexpressing cells.

Rack1 overexpression does not inhibit downstream signaling from src

As noted above, it has been reported that Rack1 can bind to and inactivate src family kinases. To explore whether this might account for the observed phenotype in the Rack1 overexpressing lines, we determined the effects on some of the downstream targets of src signaling. Two substrates of src, FAK and p130cas, have been implicated in migratory function (Cary et al., 1996; Gilmore and Romer, 1996; Klemke et al., 1998) and we initially examined if these substrates were differentially phosphorylated in our Rack1 overexpressing cell lines. To do this A5 and R8 cells were serum starved overnight, put into suspension for 2 hours, and then allowed to adhere to fg for 10 minutes. Lysates from these samples were then

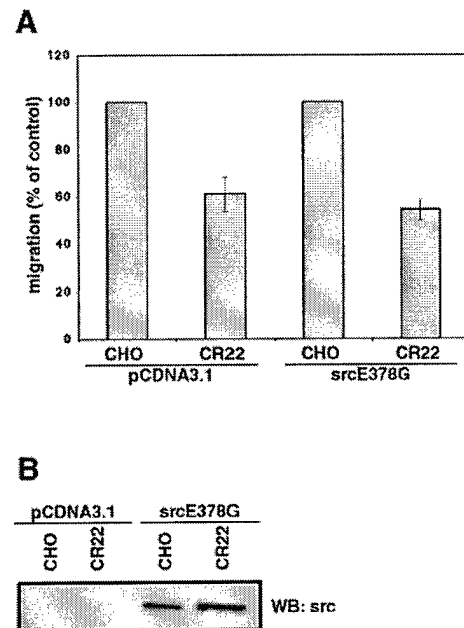


Fig. 6. Activated src does not reverse Rack1-mediated suppression of migration. (A) Wild-type CHO cells and those overexpressing Rack1 (CR22) were transfected with the pCDNA3.1 vector or a constitutively active src construct as indicated and transfectants isolated by magnetic sorting. The cells were then allowed to migrate on fn-coated Transwells and analyzed by calcein AM staining as above. (B) Expression of the transfected src construct was determined by western blotting as indicated.

immunoprecipitated with anti-FAK and anti-cas antibodies and isolated proteins analyzed by western blotting. Both the Rack1 overexpressing line and wild-type cells demonstrated increases in FAK and cas phosphorylation upon adhesion (Fig. 5A,B). Src activity has also been implicated in the upregulation of the MAP kinases. Therefore, we examined whether ERK1/2 were differentially phosphorylated and activated in Rack1 overexpressing cells. However western blotting with a phospho-ERK specific antibody suggested comparable upregulation of these kinases upon adhesion (Fig. 5C). Thus signaling events downstream of src do not appear to be disrupted in the Rack1 overexpressing cells.

As a final approach to determine if altered src function could account for the decreased motility of Rack1 overexpressing cells, we asked whether we could reverse this phenotype by transfection with an activated src construct. However, transfection of CR22 cells with srcE378G did not enhance migration to wild-type levels (Fig. 6A), despite ample expression of this src variant (Fig. 6B). Thus it appears that Rack1 overexpression did not diminish src activity in our system and this is not a mechanism for the observed migratory defects.

Inhibition of cell migration is reversed by a mutant Rack1 construct

Two sequence motifs in Rack1, one in its third WD domain and one in its sixth WD domain, are homologous to areas in other PKC-binding proteins (Ron et al., 1994). Furthermore,

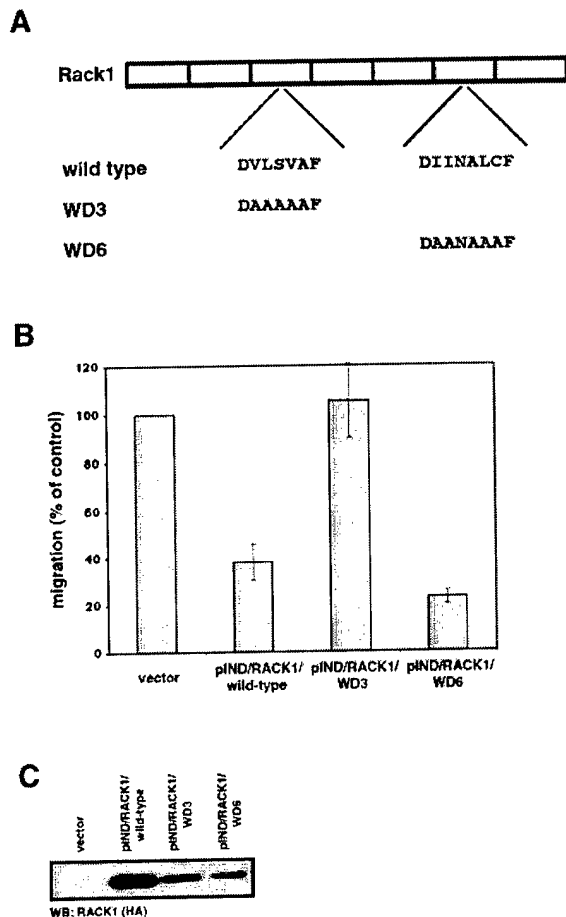


Fig. 7. Mutation of a putative PKC-binding region in Rack1 reverses its effects on cell migration. (A) The WD domain structure of wild-type Rack1 is illustrated. Putative PKC binding sites exist in the third and sixth domains and residues in these areas have been targeted by alanine substitution as indicated. Distinct constructs containing WD3 substitutions and WD6 substitutions have been generated. (B) CHO cells were transiently transfected with vector, wild-type Rack1, the WD3 variant, or the WD6 variant and isolated by magnetically sorting after 48 hours. Equal amounts were applied to fn-coated Transwells and allowed to migrate for 12 hours. Quantitation of migrated cells was by calcein staining as described above. While cells expressing wild-type Rack1 and the WD6 variant demonstrated reduced migration, vector transfectants and those expressing the WD3 variant had wild-type motility. (C) Western blotting analysis demonstrated that all constructs expressed equally well.

synthetic peptides corresponding to each of these sequences have been directly implicated in PKC binding and in downstream, PKC-dependent functional effects (Ron et al., 1994; Ron and Mochly-Rosen, 1994). Therefore, to assess the role of Rack1-PKC interactions in our observed inhibition of migration, we have generated alanine substitutions within these areas (Fig. 7A). When we overexpressed a Rack1 construct with alterations in its WD6 domain, we observed no change from those effects with wild-type Rack1 (Fig. 7B). That is migration was inhibited with respect to control transfectants. However, when we overexpressed a variant with substitutions

in its WD3 domain, we observed no inhibition of migration (Fig. 7B). All Rack1 variants were expressed to similar extents. Thus PKC interactions with a motif in the third WD domain of Rack1 appear to be important in integrin outside-in signaling, especially with regard to cell migration.

DISCUSSION

Racks have been classically defined as saturable, specific receptors that stabilize PKC isoforms in the active state and anchor them to membranes or other functional sites (Mochly-Rosen and Gordon, 1998). The data presented in this paper suggest that Rack1, a receptor for the PKC β isoform that also associates with integrins and src kinases as well (Chang et al., 1998; Liliental and Chang, 1998) is involved in regulating cytoskeletal organization and downstream events such as integrin-mediated cell migration. Rack1 thus represents another player in a cell's migratory machinery and a novel therapeutic target for regulating this process.

Unlike other proteins which interact with specific integrins (eg. β_3 -endoneurin, Shattil et al., 1995; CIB, Naik et al., 1997), we and others have shown that Rack1 appears to interact with several integrin β subunit tails. The functional studies described here examine Rack1 effects on downstream signaling mediated by the β_1 and β_3 integrins. We do not yet know if Rack1 overexpression also affects migration mediated by other integrins (β_2 and β_5). Thus it is not clear if Rack1 broadly affects integrin outside-in signaling or if some measure of specificity, which includes the β_1 and β_3 integrins, does exist. It is also of interest that we could not overexpress Rack1 to greater than twofold above endogenous levels in either the stable or transient systems. One potential explanation for this is that, like structurally homologous G-protein β subunits, Rack1 may exist as part of a greater protein complex. These additional components may be limiting and therefore affecting Rack1 expression levels.

The multiple binding partners of Rack1 suggest several possibilities whereby this protein might affect cytoskeletal structure and cell motility. First, this protein has been reported to bind to and inactivate src family kinases. Src in turn can be linked to migratory behavior via distinct pathways involving the phosphorylation of FAK and p130cas. Phosphorylation of FAK at residue 925 creates a binding site for the grb2-sos complex and induction of ERK kinase activity through the classical ras pathway. The migration of FG carcinoma cells has been shown to be dependent upon ERK activity and the subsequent phosphorylation of myosin light chain kinase and myosin light chains (Klemke et al., 1997). On the other hand, phosphorylation of p130cas creates a binding site for crk and this association has been demonstrated to be important in the migratory and invasive properties of carcinoma cells in a manner dependent upon the G protein, rac (Klemke et al., 1998). Thus Rack1 inhibition of src kinases seems an attractive hypothesis for the migratory defects we have observed. However, our Rack1 overexpressing cell lines did not demonstrate deficiencies in FAK or cas phosphorylation and MAP kinase activity (Fig. 5), and we do not believe this is a mechanism for reduced migration of our cell lines. In support of this, transfection with a constitutively active src construct did not overcome the migratory defect in Rack1 overexpressing

cells. In this regard, our results appear to differ from those of Chang et al., where reduced src activity upon Rack1 overexpression could explain their observed phenotypes such as altered growth rates of NIH 3T3 cells (Chang et al., 1998). Cell type differences might be an explanation for these discrepancies. Nevertheless we have not measured src activity directly in our cell lines and cannot discount the possibility that Rack1-mediated inhibition of src activity may limit migration by another means. In this regard, it has been demonstrated that focal adhesion turnover is reduced in cells expressing kinase inactive or myristylation-defective src constructs (Fincham and Frame, 1998). Cells with decreased focal contact turnover would be predicted to have increased adhesive strength, and consequently, decreased motility. Consistent with this, we have observed striking differences in the number of stress fibers and focal contacts between wild-type and Rack1 overexpressing cells (Fig. 3).

On the other hand, Rack1 interaction with PKC isoforms may be important in our observed phenotypes. The involvement of PKCs in several integrin-mediated functions such as spreading (Haller et al., 1998; Vuori and Ruoslahti, 1993), cytoskeletal assembly and adhesion (Haller et al., 1998; Lewis et al., 1996), migration (Derman et al., 1997; Rigot et al., 1998; Volkov et al., 1998), receptor endocytosis (Panetti et al., 1995), and FAK and MAP kinase activation (Lewis et al., 1996; Miranti et al., 1999; Rigot et al., 1998) has been well documented. Interestingly, it has been reported that a specific isoform, PKC ζ , is involved in the adhesion and chemotaxis of neutrophils (Laudanna et al., 1998). Thus, the association of Rack1 with integrin tails might enable or otherwise affect PKC isoform-specific localization. Overexpression of Rack1 might disrupt this balance, thereby contributing to the observed cytoskeletal and migratory effects. Although we have not directly analyzed its PKC binding properties, the absence of a migratory deficit in cells overexpressing the WD3 domain variant appears to support this idea. It will be interesting to identify specific residues within this targeted area that mediate these effects. It is presently not clear why cells expressing the WD6 variant did not also have this property. Peptides corresponding to this motif have also been implicated in PKC binding and the downstream functional consequences thereof (Ron et al., 1994; Ron and Mochly-Rosen, 1994). Finally, as Rack1 is composed of WD domains and these motifs have been implicated in protein-protein interactions, it is also conceivable that another unrecognized molecule may mediate our observed effects. In this scenario, Rack1 overexpression and the WD3 domain mutations might affect things such as PKC activity, substrate availability, or protein folding.

In addition to Rack1, two other Racks have been cloned and identified. These include β' -COP, an ϵ PKC receptor (Csukai et al., 1997), and Pick1, an α PKC receptor (Staudinger et al., 1995). While little is known about the structure of Pick1, Rack1 and β' -COP consist of WD repeats, a structural motif held in common with G protein β subunits. Each WD repeat consists of approximately 40 residues with a common core typically bracketed by GH (glycine, histidine) and WD (tryptophan, aspartic acid) (Neer et al., 1994). Structure determinations of WD-containing proteins suggest a propeller motif where each propeller blade consists of four antiparallel β sheets (Garcia-Higuera et al., 1998). WD repeat proteins are involved in diverse cellular functions including cytoskeletal

assembly, intracellular trafficking, mRNA splicing, transcriptional regulation and, as implicated in our studies, cell migration. It is not known if the other identified Racks, β' -COP and Pick1, can associate with integrins or are involved in integrin-mediated function. On the contrary, β' -COP has been implicated in vesicular trafficking and Golgi function (Csukai et al., 1997). Nevertheless, it is tempting to speculate that these or other Racks might function to translocate PKCs to integrins or focal contacts and assist in downstream signaling. In light of this idea, it is interesting to note that another WD-containing protein, WAIT-1, has been isolated in a yeast two-hybrid screen using the integrin β_7 tail as a bait (Rietzler et al., 1998). It is presently unknown whether WAIT-1 functions as a Rack or what the functional consequences of WAIT-1-integrin β_7 interactions might be.

In summary, we have shown that overexpression of the scaffolding protein Rack1 in CHO cells increases the number of actin stress fibers and focal contacts upon adhesion and decreases cell motility in a manner that likely involves its interaction with PKC. Identification of those signaling pathways involving Rack1 and PKC or disrupting their association with integrins might define a novel means of regulating migration in pathological situations.

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